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ISOLATION OF AN ANTI-CARCINOGENIC COMPOUND: MYRICETIN FROM *COCHLOSPERMUM RELIGIOSUM*

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ABSTRACT: Studies on plant secondary metabolites have been increasing over the last 70 years. These molecules are known to play a major role in the adaptation of plants to their environment, but also represent an important source of active pharmaceuticals. Role of primary metabolites in basic life functions such as cell division and growth, respiration, storage, and reproduction have been described in modern chemistry and biology. In biology, the concept of secondary metabolite can be attributed to Kossel. He was the first to define these metabolites as opposed to primary ones. In the present study, the bioactive secondary metabolite myricetin was identified and isolated from *in vivo* and *in vitro* tissue of *Cochlospermum religiosum* a critically endangered medicinal plant. Myricetin is a naturally occurring flavonol found in many plants like grapes, berries fruits, vegetable, herbs as well as other plants. Myricetin has wide array of biochemical properties, such as anti-neoplastic, anti-carcinogenic antioxidant activity, anti-inflammatory effects. Myricetin was isolated and identified from leaf and callus by using different techniques such as IR spectra and HPTLC.

INTRODUCTION: *Cochlospermum religiosum* is a plant which belongs to family Chochlospermaceae and commonly called as silk cotton. It is an endangered species which is widely distributed in many places of India such as plains of Uttar Pradesh, Bihar, West Bengal, Orissa, M.P, Maharashtra, Gujarat and Rajasthan. This species contains varied amount of flavonoids. Flavonoids are small molecular secondary metabolites synthesized by plants with various biological activities.

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Due to their physical and biochemical properties, they are capable of participating in plants' interactions with other organisms (microorganisms, animals and other plants) and their reactions to environmental stresses [1].

Flavonoids occur universally in higher plants but are uncommon in cryptogams. They are common constituent of many medicinal plants. Flavonoids in plants can function as color definition and attractant to pollinators and seed dispersers, as antioxidant to protect plants against UV-radiations, in inducible defense against bacterial and fungal attack [2]. For human, several health beneficial properties of dietary flavonoids are recognized for their antioxidant and anti-proliferative effects which may protect the body from various diseases such as cancer, cardiovascular disease, inflammatory and antimicrobial significance.

Myricetin is a naturally occurring flavonoid found in many plants including *Cochlospermum religiosum*. Myricetin has been receiving the most attention because of its wide array of biochemical properties, such as anti-neoplastic, anti carcinogenic, antioxidant activity, anti-inflammatory effects and transiently reduces established neuropathic pain behaviour [3, 4]. Myricetin has greater antiradical activity than other flavonoids and it scavenge oxygen radical and inhibits lipid peroxidation. It is increasingly discovered for its potential analgesic effects in mostly inflammatory and acute pain states and has anti neoplastic and anti-inflammatory effects.

Previous studies have demonstrated that myricetin has anti cancer effects against several types of cancer, including hepatocarcinoma, skin carcinoma and pancreatic cancer. The anticancer property of myricetin has been attributed mainly to its antioxidant action. Comparative study on the ability of the myricetin and quercetin to modulate the oxidative DNA damage induced by hetrocyclic amines [5].

Though these compounds are generally extracted from plant parts, the plant tissue culture technique has widened the scope and opened a new front for the production of secondary metabolites. Now through this method large number of medicinal plants has been produced *viz. Stemona tuberose* [6], *Chrysanthemum morifolium*[7], *Solidago virgaurea* [8] and *Cocculus hirsutus* [9]. Flavonoid can be produced by using different biotechnical approaches, such as callus culture, cell suspension culture or organ culture. In the presented section we used sample from *in vitro* callus culture to compare with normal leaf. HPLC is increasingly used for the analysis of plant extracts these days. A "fingerprint" chromatogram produced by qualitative analysis under standard conditions can be very useful for quality control of phytochemicals.

HPLC can also be a useful tool in chemosystematics helping, for example, to characterize species on the basis of their secondary metabolite contents. As myricetin is considered as an active ingredient of the plant, the present study was undertaken for isolation, identification and quantitative estimation of myricetin content from various plant parts and callus tissue of *Cochlospermum religiosum* through various techniques such as HPTLC and IR specrtophotometry. The isolation and identification of myricetin by HPLTLC studies have also been reviewed by many authors [10, 11].

MATERIAL AND METHODS:

During the present studies, myricetin content was estimated by HPTLC.

(i) Preparation of extract:

In vivo and *in vitro* samples:

Mature plant parts (leaves) of *Cochlospermum religiosum* were collected and were washed with tap water to remove dust and dried in shade. Six week old callus tissue of *Cochlospermum religiosum* grown on MS-medium supplemented with BAP (2.0 mg/l) and NAA (1.0 mg/l) was dried in an oven at 100℃ for 15 min. to inactivate enzymes, followed by 60℃ till a constant weight was achieved. Tissue samples were powdered and used for extraction.

The *in vivo* and *in vitro* samples of the plant were dried in the dark at room temperature, powdered and extracted by soxhlet extraction method using methanol as solvent. Afterward, the extract was filtered using w man filtered paper-42 and then the solvent was distilled under reduced pressure in rotatory vaccume evaporator until the extracts became dry.

(ii) Reagents and other materials:

Myricetin (sigma Aldrich), Methanol, Ethyl Acetate, Formic Acid (all reagents of analytical grade, E-Merck) and silica gel F_{254} precoated TLC aluminium plates (E-Merck).

(iii) Preparation of standard and sample solution:

In standard preparation, 5mg of myricetin was dissolved in 5ml methanol in 10ml vol. flask. In sample preparation weigh 5gm of sample in a 50ml standard flask. Add 20ml methanol and shake well and sonicate for 10 min, make the final volume to 50ml with methanol. Filter and evaporate the solution and prepare residue. Dissolve this residue in 5ml of methanol. This is test solution.

(iv) Development of HPTLC Technique:

A densitometric HPTLC analysis was performed with methanolic extract for the development of characteristic fingerprint profile which may be used for quality evaluation and standardization of the drug. The TLC plate was activated by placing in an oven at the temperature of 120℃ for 20 min. 10µl of extract was spotted on precoated silica gel G HPTLC plates (cut to 10x10 cm) with the help of CAMAG Linomat 5 applicator maintaining a distance of 15 mm from the edge of TLC plate. Precoated HPTLC plate of silica gel 60 G as stationary phase was used.

Linear ascending development was carried out in twin trough glass chamber (20x20 cm) saturated with mobile phase [Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.6:0.4)] for 5 min at 60℃ temperature. The developed plates were scanned using TLC scanner 3 and spots were visualized under UV Light at 254nm, 366nmnm and under visible light at 540nm. Deuterium and tungsten lamps were the source of radiation utilized. Each of the fluorescent spots coincide with those of standard references compound of myricetin were marked.

The marked spots were scrapped and collected separately along with the silica gel 'G' and eluted with ethanol. Each of the elutes was then crystallized with chloroform. The compounds thus isolated were subjected to infra red spectral studies.

Myricetin estimation in *Cochlospermum religiosum:*

Instrument: HPTLC system equipped with a sample applicator device Canag Linomat 5. Camag twin trough chamber, Camag TLC scanner.

Stationary phase: TLC Al sheet silics gel $60F_{254}$ precoated cut to 10 x 10 cm.

Mobile phase: Toulene: Ethyl Acetate: Formic acid: Methanol (3:3:0.6:0.4)

Standard: Myricetin 1mg/ml

Sample: *C.religiosum in vivo* and *in vitro* plant extract 10mg/ml

Development distance: 80mm

Scanning wavelentgth: 254nm, 366nm and 540 nm

Measurement mode: Tungsten Lamp. (After Derivatisation with AS)

(v) Quantification of Myricetin in different samples:

The calibration curve was prepared by plotting concentration (µg/spot) versus peak area corresponding to each spot. So, the amount of myricetin in different samples was calculated using the respective calibration curve. The plate were developed and scanned at 254,366 and 540 nm. The peak area and observation spectra were recorded. Concentration of the metabolite in the sample was calculated by considering the sample initially taken and diluted factors.

Quantitative evaluation of the plate was performed in the absorption mode at 254 nm, with the following conditions slit width 6.00 x 0.30mm, micro scanning speed 20 mm/s and data resolution 100 um/step. (Executed by- Arbro pharmaceuticals, Delhi)

RESULT AND DISCUSSION:

In the present study, the bioactive secondary metabolite myricetin was identified and isolated from *in vitro* and *in vivo* tissue samples. The purity of myricetin bands in the sample extracts was confirmed by comparing the absorption spectra at start, middle and end position of the band. Under the chromatographic condition described above, the Rf's value of myricetin was determined to be approximately 0.63 for *Cochlospermum religiosum*.

The Rf's obtained for this plant extracts closely replicate the Rf's found for myricetin (working standard for myricetin), thus making it a significant fingerprint parameter identifying it as myricetin. TLC fingerprint image of standard myricetin with plant extracts is shown at different wave length **Plate 1 Fig ABC**. The chromatogram of standard myricetin i.e peak are shown in **Plate-2, Fig A** and that of myricetin in *Cochlospermum religiosum* are shown in **Plate 2 Fig B & C**. The respective Rf's obtained for each sample is shown in (**Table 1**).

Spectral comparison brings out the overlaid spectra between the selected samples at a selected wavelength which in the present case was 254nm thus facilitating a match between the spectra of the

plant extract and that of the working standard. Thus the flavonoid myricetin has been further identified by using IR spectra. The characteristic IR spectral peaks were found to be super imposable with those of their respective standard reference compounds of myricetin **Plate 3**.

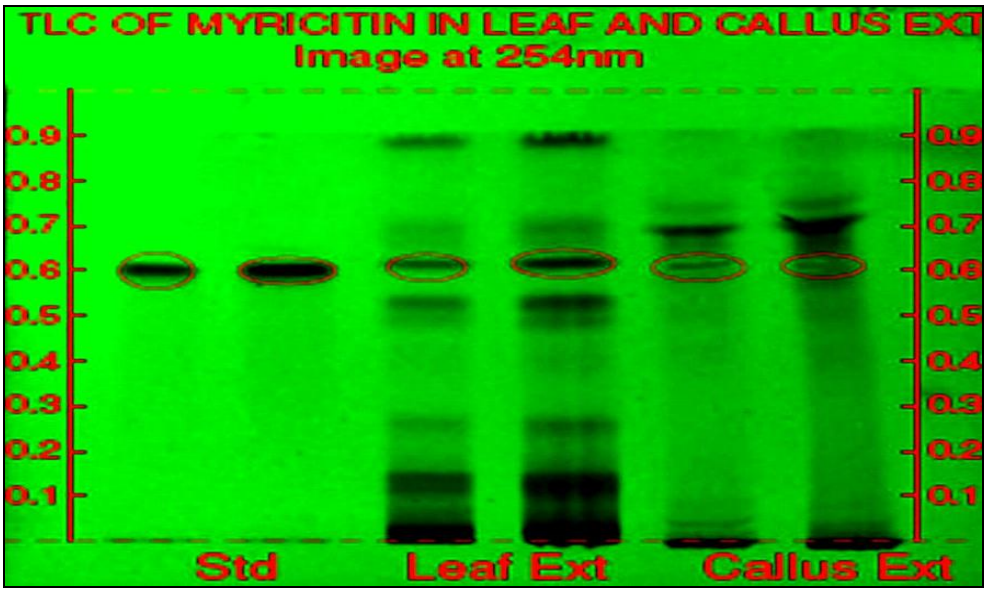
When isolated myricetin was subjected to HPTLC, it showed Rf value 0.63 which coincide with that of standard myricetin **Plate 2, Fig A**. Thus, myricetin was detected in all samples. In both samples leaf had maximum amount of total myricetin (0.1224%) while minimum amount was present in methanolic extract of callus (0.0731) **Table 2 Plate 2, Fig B&C**.

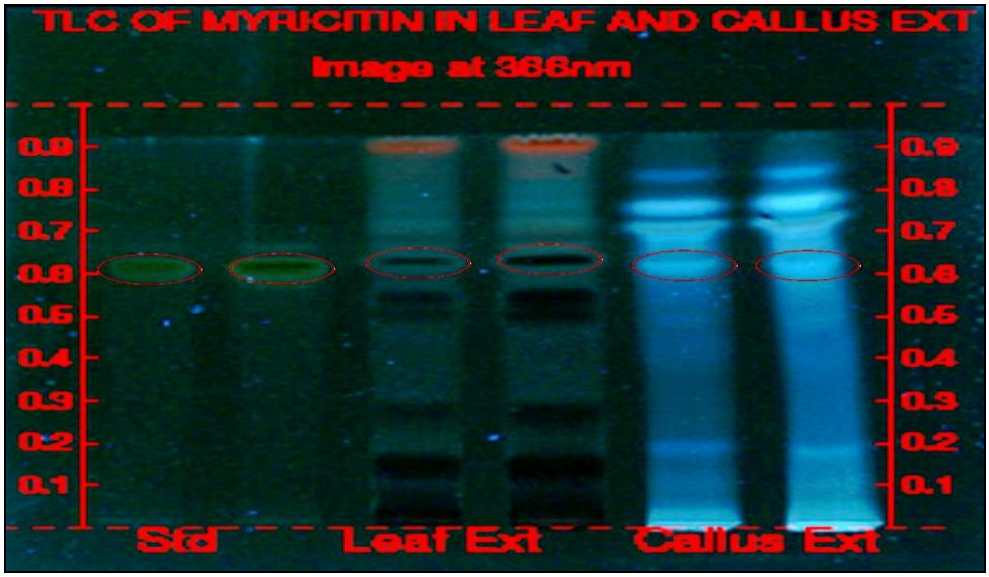
TABLE 1: Rf VALUES OF MYRICETIN OF *COCHLOSPERMUM RELIGIOSUM*

Parameteres	Myricetin Standard	Leaf Sample	Callus Sample
Standard Rf	0.56	0.60	0.57
Max. Rf	0.63	0.64	0.60
End Rf	0.65	0.66	0.65

TABLE 2: CALCULATION AND CALIBERATION OF MYRICETIN OF *COCHLOSPERMUM RELIGIOSUM*

Sample	Leaf extract	Callus extract
Area of sample	4256.1	1702.8
Area of standard	11542.1	11554.1
Wt. of standard (mg)	4.94	4.94
Wt. of sample	7288.3	4880.8
Standard potency	98.00%	98.00%
Result in %	0.12246832	0.07316625





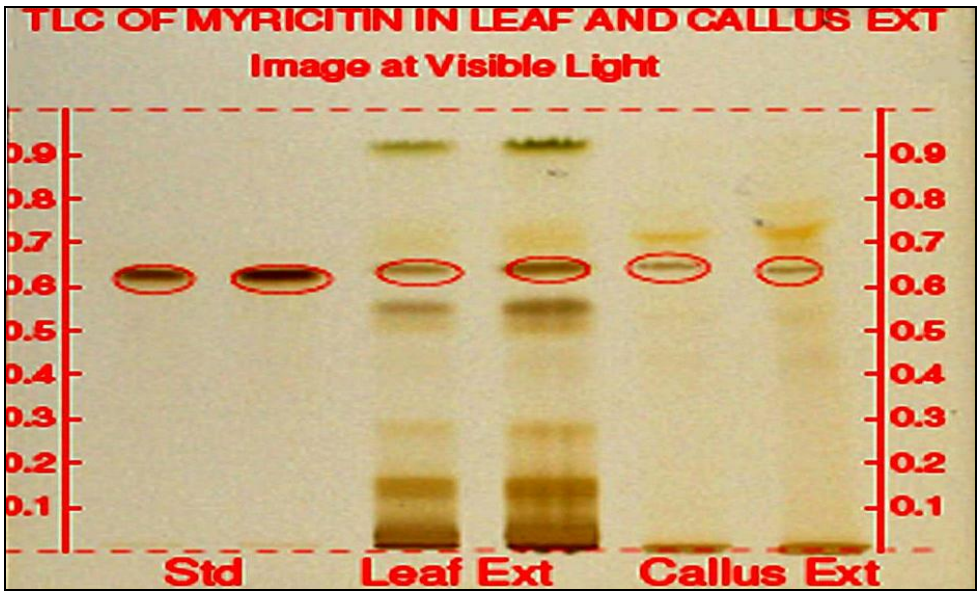


PLATE: 1

FIG: TLC PLATES SHOWING PRESENCE OF MYRICETIN IN ISOLATED *IN VIVO* AND *IN VITRO* SAMPLES AT DIFFERENT WAVELENGTH (254nm, 366nm and 540nm).

Abbrevations:
L=Leaf,
C=Callus,
Std= Standard

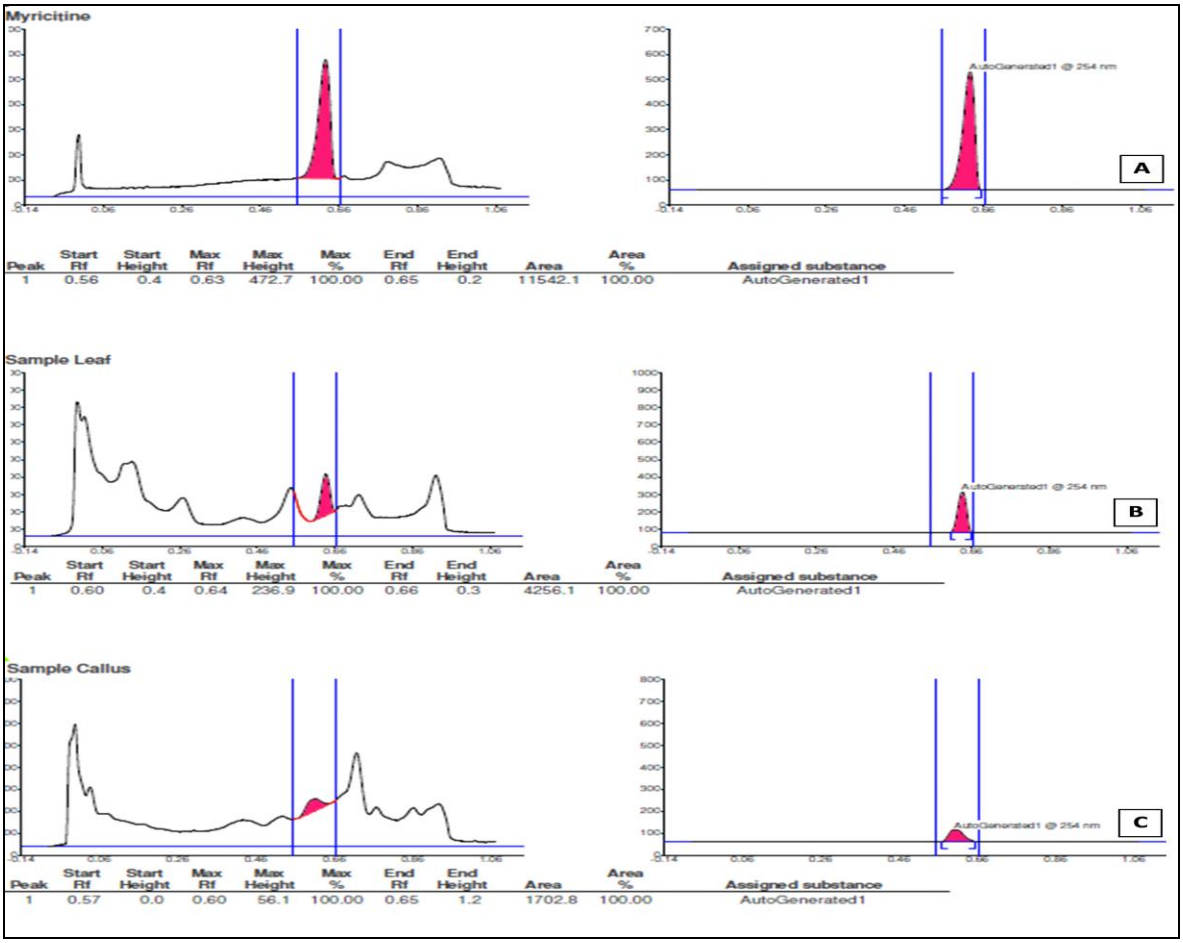


PLATE: 2

FIG. A-C: HPTLC CHROMATOGRAMS OF MYRICETIN.
FIG. A: CHROMATOGRAM OF STANDARD MYRICETIN.
FIG. B: CHROMATOGRAM OF MYRICETIN IN CALLUS.
FIG. C: CHROMATOGRAM OF MYRICETIN IN LEAVES

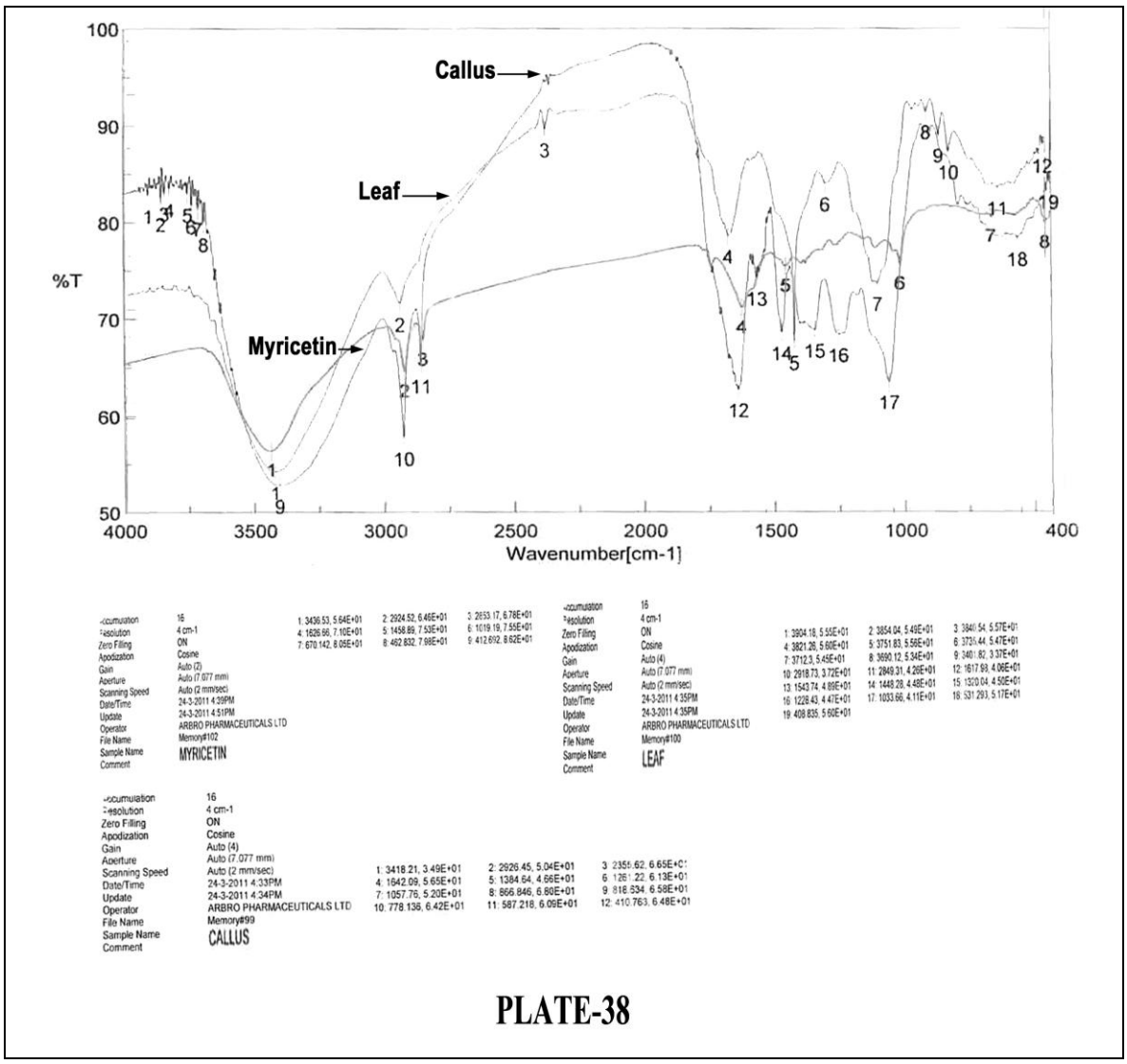


PLATE: 3

SUPERIMPOSED IR SPECTRA OF MYRICETIN IN ISOLATED *IN VIVO* AND *IN VITRO* SAMPLES OF *C. RELIGIOSUM* WITH STANDARD MYRICETIN

CONCLUSION: HPTLC is an enhanced form of thin layer chromatography which is rapid, comparatively simple, robust and extremely versatile. It can not only confirm but also establish identity. All the results concerning the present investigation revealed the following conclusions. The Rf value of standard myricetin was found to be 0.63 which coincide with Rf value of leaf and callus (0.64 and 0.60 respectively) confirming its identity.

These results further show the presence of myricetin in all *in vivo* and *in vitro* samples. Variation in myricetin content in *in vitro* and *in vitro* samples in *Cochospermum religiosum* was observed, leaf had maximum amount of total myricetin (0.1224 %) while minimum amount of myricetin was present in callus (0.0731%). IR spectral peaks were found to be superimposible with those of their respective standard references compound of myricetin confirming presesnce of myricetin in all samples.

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